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A VERSATILE AUTOMATED SYSTEM FOR MEASURING BLOOD CHOLINESTERASE ACTIVITY

by

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March 1972

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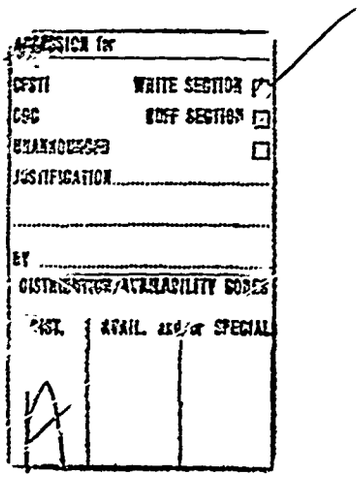

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13. ABSTRACT

(U) A multipurpose automated blood ChE system is described. Venous blood samples may be assayed directly with auto dilution or capillary blood samples may be assayed after manual dilution. Repeatability values and correlation coefficients obtained from enzyme assays comparing autodilution with manual dilution were excellent.

14. KEYWORDS

Automation
Cholinesterase
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Task 1W062116AD1904

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FOREWORD

The work described in this report was authorized under Task 1W062116AD1904, Techniques of Evaluating Effects of Chemicals, New Methods for Biological Assays. The experimental work was started in February 1971 and completed in September 1971. The experimental data are contained in notebooks MN 2368 and MN 2422.

The volunteers in these tests are enlisted US Army personnel. These tests are governed by the principles, policies, and rules for medical volunteers as established in AR 70-25.

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences–National Research Council.

DIGEST

This report describes a multipurpose automated system for measuring blood ChE activity. This system may be used in either an autodilution or manual dilution mode. Repeatability studies demonstrated that the precision of whole blood, plasma, or red cell ChE measurements, expressed as coefficients of variation, lies within ±2%. Correlation coefficients obtained from analyses of whole blood, plasma, and red cell data, comparing autodilution with manual dilution, are 0.98, 0.99, and 0.99, respectively. Red cell and plasma activity curves are linear over a wide range of dilutions. In man and ten other animal species tested, the sulfhydryl content of red cells increases as ChE activity decreases.

CONTENTS

A VERSATILE AUTOMATED SYSTEM FOR MEASURING BLOOD CHOLINESTERASE ACTIVITY

I. INTRODUCTION.

Numerous pesticides, drugs, and chemical agents manifest their action by depressing levels of cholinesterase (ChE) in blood. Emergency aid must be predicated on symptoms, but laboratory measurements of blood enzyme are essential, particularly for determining response to drug therapy.

Cholinesterase methods are rapidly becoming established as routine laboratory procedures. Numerous manual and automated techniques have been proposed. Although the manual methods are generally reliable for serum or plasma, they are generally not as reliable for red cell measurements and are considerably slower than automated methods. A particular disadvantage of the manual colorimetric techniques is the high nonenzymatic absorbancy which occurs with blood and tissue homogenates.

The purpose of this report is to present a versatile and accurate automated system for measuring blood ChE. A secondary purpose is an attempt to standardize experimental conditions for reporting enzyme activity. This system is designed for the multiple requirements of a clinical laboratory. An attempt has also been made to eliminate some of the factors that result in differences among automated ChE systems, as described in our recent report.[1] For this reason, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) is added to the sample stream as recommended by Humiston and Wright.[2] This method incorporates both an autodilution mode which eliminates manual pipetting, and a manual mode, in which case capillary samples of blood are diluted manually prior to assay. Good precision and identical values are obtained by both modes of operation. The method correlates with the widely accepted ΔpH (Michel)[3] manual method and Hestrin (Groff)[4] automated method.

II. MATERIALS AND METHODS.

A. Reagents.

1. Saponin, 0.01%. 0.1 gm of saponin in a liter of distilled water containing 5 ml of Brij 35.

2. Tris Buffer, pH 8.2, 0.05 M. 6.640 gm of sodium chloride and 6.050 gm of Tris in approximately 950 ml of distilled water containing 5 ml of Brij 35. Adjust pH to 8.2, using 50% hydrochloric acid. Dilute to a liter with water.

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[1] Ellin, R. I., Groff, W. A., and Kaminskis, A. Automated Blood Cholinesterase System. A Novel Interaction and Recommendations. (Prepared for publication, 1971.)

[2] Humiston, C. G., and Wright, G. J. An Automated Method for the Determination of Cholinesterase Activity. Tox. Appl. Pharmacol. *10*, 467 (1967).

[3] Michel, H. O. An Electrometric Method for the Determination of Red Cell and Plasma Cholinesterase Activity. J. Lab. Clin. Med. *34*, 1564 (1949).

[4] Groff, W. A., Mounter, L. A., and Sim, V. M. A Multichannel Analytical System for Continuous Monitoring of Blood Cholinesterase. Automation in Analytical Chemistry. Technicon Symposium. Vol 1. pp 498-502 (1966). Mediad, Inc., New York, New York.

3. DTNB Buffer, 8.4×10^{-4} M. 0.3326 gm of DTNB in a liter of Tris buffer, pH 8.2.

4. AcSChI, 1.005×10^{-2} M. 0.2905 gm of acetylthiocholine iodide in 100 ml of distilled water. (The final concentration in the automated system is 2×10^{-3} M.)

5. Brij 35. Wetting agent obtained from Technicon Corporation.

6. Glutathione, 100 μmoles/ml. 0.3073 gm of glutathione in 10 ml of distilled water. A series of glutathione standards containing 6.25, 12.5, 25, 50, and 100 μmoles/ml, respectively, is assayed daily.

B. Multipurpose Automated Method.

The figure shows the flow diagram of the ChE system which incorporates an autodilution capability. The sample is first mixed with 0.01% saponin in water, resulting in a 1:26.8 dilution. An aliquot of the diluted sample is mixed first with DTNB, buffered at pH 8.2. Acetylthiocholine, in a final concentration of 2×10^{-3} M, is combined with the sample stream immediately before the mixture enters the 37°C heating bath. After incubation, the sample stream is dialyzed against Tris buffer (pH 8.2), using a type C cellophane membrane. The incubation time at 37°C, measured from the point at which substrate is added to the sample stream until the sample stream exits from the dialyzing plate, is approximately 6 min. The absorbance of the recipient stream is measured at 420 nm in a 15-mm flow cell. A separate blank run should be made in the absence of substrate to correct for sulfhydryl compounds present in the sample material; human plasma and red blood cell blank readings show little variation from individual to individual (plasma absorbance blank, 0.003 ±0.001, RBC absorbance blank, 0.071 ±0.005).

1. Autodilution.

A portion of undiluted red blood cells or plasma is automatically sampled (tube A in flow diagram), diluted, and an aliquot taken into the analytical system for assay. A wide range of dilutions can be obtained by selecting the appropriate sizes of sample and/or diluent pump tubes. Human red blood cells and plasma are diluted 1:26.8. Saponin (0.01%) in distilled water is the diluent lysing solution. Isotonic saline is pumped to the Sampler II wash receptable.

The separation between recorded peaks when using the autodilution system was not optimum because the aliquot line (B) draws liquid at all times and no air enters this line between samples. Without an air bubble to scrub the aliquot tube between samples, wash time must be prolonged to improve separation of peaks.

Improved separation of peaks was accomplished by alternating cups containing diluent with cups containing red blood cells or plasma on the sample plate. Operating the system at 60/hour results in an effective sampling rate of 30/hour and excellent peak separation.

2. Manual Dilution.

A portion of manually diluted red blood cells or plasma (1:26) is sampled directly into the analytical system by connecting the sample line to the aliquot pump tube (tube B in flow diagram, figure). A sample volume of 20 μl is adequate for manual dilution prior to assay. Manually diluted samples may be assayed at the rate of 60/hour without the use of wash cups between samples.

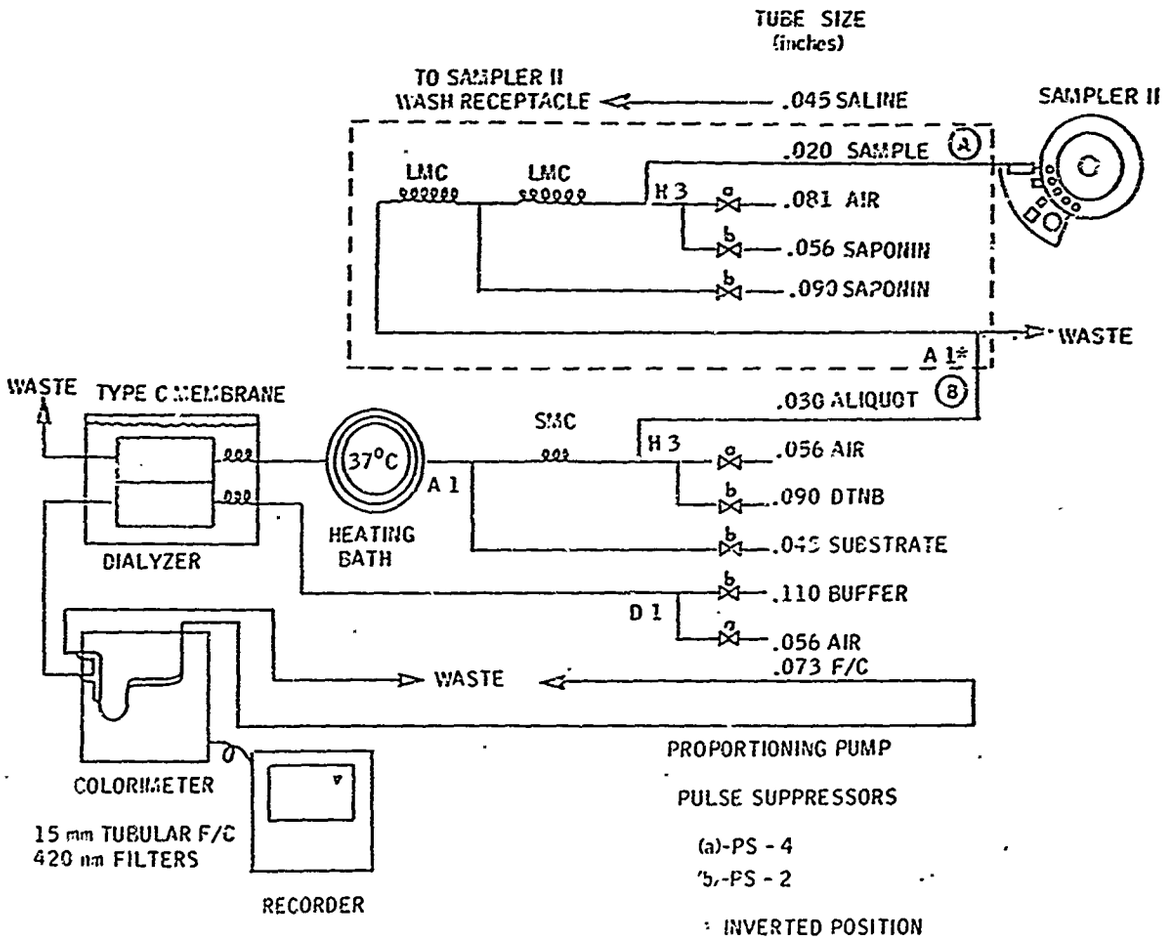

Figure. Autodilution Manifold for Measuring ChE Activity

For sampling manual dilutions, disconnect Sampler II from tube Ⓐ and connect to tube Ⓑ. Discontinue pumping reagents in that portion of the manifold inclosed by dashed line.

C. Comparison of Autodilution and Manual Dilution.

Six samples of human whole blood, plasma, and erythrocytes were assayed at a rate of 60/hour with wash cups between samples. The same six samples were diluted manually and assayed at 60/hour without wash cups. Results appear in the appendix of this report. A 10^{-1}M glutathione standard was similarly assayed by each technique.

D. Repeatability of ChE Assay by Manual and Autodilution Techniques.

Human whole blood, plasma, and red blood cell samples were assayed by manual dilution and autodilution. A series of four values was obtained by each technique. The results are presented in the appendix.

E. Enzyme Dilution Curves.

A pooled red blood cell sample and a pooled plasma sample were each diluted serially with 0.01% saponin to give the following concentrations: 50, 25, 12.5, 6.3, 3.1, and 1.6%. Both series of dilutions and the undiluted samples were assayed by the autodilution technique.

F. Relative Sulfhydryl Content of Red Blood Cells of Various Species.

Packed red cells which had been washed once with isotonic saline were assayed in the absence of substrate. The cells were diluted manually and hemolyzed prior to sampling. Human red cells were diluted 20-fold, monkey red cells 10-fold, and all other species 5-fold. Glutathione solutions were assayed in the same manner and absorbancies plotted versus concentration to obtain a standard curve.

III. RESULTS.

A comparison of human whole blood, plasma, and red cell ChE values obtained by autodilution and manual dilution shows good agreement (table A-I). Correlation coefficients obtained from analyses of whole blood, plasma, and red blood cell data by both methods are 0.98, 0.99, and 0.99, respectively. Repeatability data for the autodilution and manual dilution modes of the multipurpose system were obtained by assaying a series of six samples four times each over a 6-hour interval. Calculated coefficients of variation for either whole blood, plasma, or red cell ChE vary by less than ±2% (tables A-II and A-III).

Linear activity curves were obtained after diluting the red blood cells and plasma with 0.01% saponin (table I).

Table II shows the red blood cell ChE activity and sulfhydryl (SH) concentration for man and ten other animal species. The ratio of enzyme activity to concentration of SH in man is approximately 1; that in the cat and rat, the lowest value is 0.07.

IV. CONCLUSION.

A multipurpose method is presented for measuring ChE activity. The system can be used in either an autodilution or manual dilution mode, making it possible to assay either venous or capillary blood samples. In either mode, the results of whole blood, red cell, and plasma ChE assays

Table I. Cholinesterase Activity of Red Blood Cells and Plasma Diluted With 0.01 Percent Saponin

Dilution	Red cell ChE	Plasma ChE
%	μmoles/ml/min	μmoles/ml/min
Undiluted	11.21	4.51
50	5.63	2.21
25	2.88	1.11
12.5	1.32	0.54
6.3	0.60	0.28
3.1	0.30	0.13
1.6	0.14	0.07

Table II. Sulfhydryl Content of Animal Erythrocytes

Species	Red cell ChE activity*	μmoles SH/ml red blood cells	A/B	Optimum AcSChI concentration
	A	B		M
Human	12.6	13.7	0.92	2×10^{-3}
Monkey	7.1	10.6	0.67	2×10^{-3}
Pig	4.7	10.1	0.47	10^{-3}
Goat	4.0	9.9	0.40	2×10^{-3}
Sheep	2.9	10.6	0.27	2×10^{-3}
Mouse	2.4	11.7	0.21	2×10^{-3}
Dog	2.0	12.3	0.16	2×10^{-2}
Rabbit	1.7	12.0	0.14	5×10^{-3}
Guinea pig	2.7	20.7	0.13	2×10^{-3}
Cat	1.5	22.7	0.07	5×10^{-3}
Rat	1.7	23.9	0.07	5×10^{-3}

*μmoles/ml/min at optimum substrate concentration.

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APPENDIX

TABLES

Table A-1. Comparison of ChE Activities Obtained by Autodilution and Manual Dilution

Biological fluid	Sample	Activity	
		Autodilution (1:26.8)	Manual dilution (1:26)
		μmoles/ml/min	μmoles/ml/min
Whole blood	1 –	10.0	9.5
	2 –	8.2	7.9
	3 –	10.2	9.7
	4 –	9.4	8.9
	5 –	11.0	10.8
	6 –	9.2	9.2
Mean ± 2 SD		9.7 ± 1.9	9.3 ± 1.9
Plasma	1 –	6.1	6.0
	2 –	3.5	3.4
	3 –	4.3	4.1
	4 –	4.0	4.0
	5 –	5.8	5.8
	6 –	4.9	4.7
Mean ± 2 SD		4.8 ± 2.1	4.7 ± 2.1
Red blood cells	1 –	12.2	12.0
	2 –	12.4	11.9
	3 –	14.2	14.1
	4 –	14.5	14.4
	5 –	14.7	14.5
	6 –	13.0	13.1
Mean ± 2 SD		13.5 ± 2.2	13.3 ± 2.3

Table A-II. Repeatability of ChE Measurements by Autodilution

Biological fluid	Activity				Coefficient of variation
	μmoles/ml/min				
Whole blood:					
Sample					
1	10.0	10.4	10.0	10.1	0.016
2	8.2	8.4	8.4	8.6	0.021
3	10.2	10.4	10.4	10.2	0.009
4	9.4	9.5	9.6	9.1	0.022
5	11.0	11.1	11.2	11.3	0.014
6	9.2	9.4	9.4	9.2	0.013
Plasma:					
Sample					
1	6.1	6.1	6.1	6.3	0.015
2	3.5	3.4	3.5	3.4	0.011
3	4.3	4.1	4.4	4.2	0.028
4	4.0	4.0	4.1	4.1	0.015
5	5.8	5.9	5.9	5.8	0.005
6	4.9	4.8	5.0	4.7	0.023
Red blood cells:					
Sample					
1	12.2	12.3	12.5	11.9	0.020
2	12.4	12.0	12.7	12.0	0.028
3	14.2	14.1	14.3	14.4	0.009
4	14.5	14.4	14.7	14.2	0.017
5	14.7	14.2	14.9	14.2	0.013
6	13.0	12.9	13.1	13.4	0.016

Table A-III. Repeatability of ChE Measurement by Manual Dilution

Biological fluid	Activity				Coefficient of variation
	μmoles/ml/min				
Whole blood:					
Sample					
1	9.5	9.4	9.3	9.3	0.010
2	7.9	8.0	7.9	7.9	0.009
3	9.7	9.9	9.8	9.7	0.011
4	8.9	9.0	9.0	9.0	0.003
5	10.8	10.8	10.7	10.6	0.008
6	9.2	9.2	9.2	9.2	0.003
Plasma:					
Sample					
1	6.0	6.1	6.1	6.1	0.007
2	3.4	3.5	3.4	3.4	0.012
3	4.1	4.1	4.1	4.2	0.010
4	4.0	4.1	4.0	4.0	0.007
5	5.8	5.9	5.8	5.8	0.009
6	4.7	4.8	4.6	4.8	0.015
Red blood cells:					
Sample					
1	12.0	11.9	11.7	11.7	0.014
2	11.9	11.9	11.8	11.8	0.007
3	14.1	14.0	13.8	13.8	0.013
4	14.4	14.2	14.0	14.1	0.012
5	14.5	14.3	13.9	14.1	0.015
6	13.1	13.2	12.7	12.8	0.017